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Review

Tracking fetal development through molecular analysis of maternal biofluids ☆

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ABSTRACT

Current monitoring of fetal development includes fetal ultrasonography, chorionic villus sampling or amniocentesis for chromosome analysis, and maternal serum biochemical screening for analytes associated with aneuploidy and open neural tube defects. Over the last 15 years, significant advances in noninvasive prenatal diagnosis (NIPD) via cell-free fetal (cff) nucleic acids in maternal plasma have resulted in the ability to determine fetal sex, RhD genotype, and aneuploidy. Cff nucleic acids in the maternal circulation originate primarily from the placenta. This contrasts with cff nucleic acids in amniotic fluid, which derive from the fetus, and are present in significantly higher concentrations than in maternal blood. The fetal origin of cff nucleic acids in the amniotic fluid permits the acquisition of real-time information about fetal development and gene expression. This review seeks to provide a comprehensive summary of the molecular analysis of cff nucleic acids in maternal biofluids to elucidate mechanisms of fetal development, physiology, and pathology. This article is part of a Special Issue entitled: Molecular Genetics of Human Reproductive Failure.

1. Introduction

Non-invasive monitoring of fetal development in vivo typically consists of measurement of fundal height, anatomic evaluation by fetal ultrasonography, and maternal serum biochemical screening [1]. Genetic analysis of amniotic fluid components or placental tissue obtained via chorionic villus sampling has traditionally been utilized for prenatal diagnosis. The disadvantage of these current invasive methods for prenatal diagnosis is the risk of fetal loss. The pursuit of safe and non-invasive prenatal diagnosis has been the focus of many investigators over the last 15 years.

Cell-free fetal DNA (cffDNA) in the serum and plasma of pregnant women was first described in 1997 [2]. CffDNA has also been demonstrated in other maternal biofluids, including urine, cerebrospinal fluid (CSF), and peritoneal fluid [3–6]. Recently, investigators have begun to utilize new techniques to mine the previously discarded amniotic fluid supernatant for real-time information about fetal development and global gene expression. Significantly larger quantities of cffDNA are present in amniotic fluid than in maternal serum [7]. Recent investigations of cffmRNA in amniotic fluid have suggested that fetal nucleic acids present in amniotic fluid can provide real-time information about fetal disease, physiology, and development [8–12]. This review seeks to provide a comprehensive summary of discoveries over the last 15 years that have increased our understanding of fetal development, physiology, and pathology through analysis of maternal biofluids. We also examine the present and future clinical applications for prenatal diagnosis of genetic disorders and detection of normal and pathological fetal development via maternal biofluids.

2. Fetal nucleic acids in maternal plasma, serum, and whole blood

2.1. Intact fetal cells

The isolation of intact fetal nucleated cells from maternal blood for prenatal diagnosis has been extensively studied [13]. Unfortunately, this approach has limited clinical utility due to the low number of fetal cells found in maternal circulation (approximately 1 fetal cell/mL of maternal blood in euploid fetuses), which results in low sensitivity, specificity, and reproducibility [14]. A multicenter trial sponsored by NIH reported a 41% sensitivity for detection of male fetal cells in maternal blood, and a 74% detection rate for aneuploidy; false-positive rates were 11% for fetal sex and 0.6–4% for aneuploidy [15]. Thus, alternative approaches to prenatal diagnosis through analysis of maternal blood were sought. Fig. 1 depicts a timeline of key discoveries in this field.

Abbreviations: BAC, Bacterial artificial chromosome; cff, Cell-free fetal; CLIA, Clinical Laboratory Improvement Amendments; CGH, Comparative genomic hybridization; CAH, Congenital adrenal hyperplasia; CRH, Corticotropin-releasing hormone; DMR, Differentially methylated region; HELLP, Hemolysis, elevated liver enzymes, and low platelets; IUGR, Intrauterine growth restriction; MALDI-TOF, Matrix Assisted Laser Desorption/Ionization Time-of-Flight; MPSS, Massively parallel shotgun sequencing; MeDiP, Methylated DNA immunoprecipitation; NIPD, Noninvasive prenatal diagnosis; PCR, Polymerase chain reaction; SNP, Single-nucleotide polymorphism; SNAP, Standardized NanoArray PCR; TUNEL, Terminal UdTP nuclear end labeling

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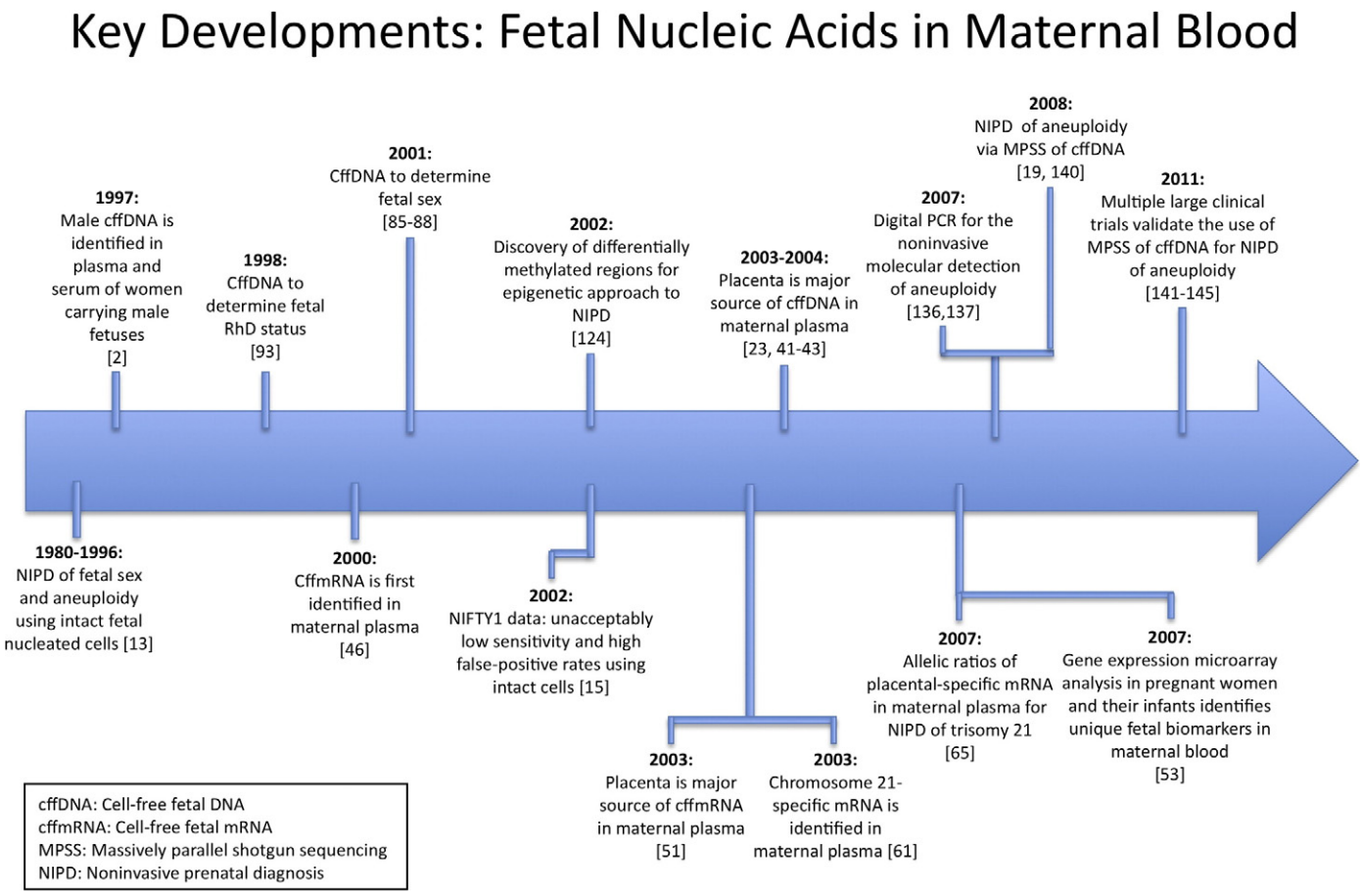

Fig. 1. Timeline of major discoveries involving fetal nucleic acids in maternal blood.

2.2. Cell-free fetal DNA (cffDNA)

The discovery of cffDNA in the maternal circulation was prompted by the presence of tumor-associated oncogene mutations and microsatellite alterations in the plasma and serum DNA of women with cancer [16]. Inspired by this work, Lo et al. hypothesized that a fetus might also release cffDNA into the maternal circulation and demonstrated the presence of male cffDNA sequences in the plasma and serum of women carrying male fetuses [2]. Significantly more fetal DNA is present in the serum of pregnant women compared with fetal DNA extracted from the cellular fraction of maternal blood [17]. CffDNA in maternal plasma comprises approximately 10% of the total cell-free DNA (range 3–19%) [18,19]. Cff nucleic acids are stable in the maternal circulation, likely due to their association with placenta-derived microparticles that protect them from nuclease degradation [11,20–22]. CffDNA increases as gestation advances, with a 21% weekly increase in the first trimester [23], a slower rate of rise in the second trimester, and a sharp increase during the last eight weeks of pregnancy [18,24–28]. Cff DNA is rapidly cleared from maternal circulation after delivery, with a half-life of approximately 16 min [29,30]. To maintain a steady state with such a short half-life, fetal DNA must be liberated continuously in large quantities into maternal circulation. Compared to analysis of intact fetal nucleated cells in maternal blood, which often requires the use of sophisticated cell enrichment procedures, analysis of cffDNA in maternal plasma and serum is rapid, reproducible, and possible to carry out on a large scale. While cffDNA in maternal plasma is reported to be stable at −20°C for ≥4 years [31], there are compelling data to suggest that the duration of specimen storage may affect the concentration of cffDNA extracted from archived maternal serum. Lee et al. observed a degradation rate of −0.66 genome equivalents (GE)/mL of cff DNA per month in samples frozen at −20 °C [32].

2.2.1. Tissue origin of fetal DNA

The placenta, fetal hematopoietic cells, and the fetus itself have all been considered as possible sources of fetal DNA in the maternal circulation [33]. CffDNA may be liberated into the maternal circulation by destruction of fetal cells that have crossed the placenta. Terminal UdTP nuclear end labeling (TUNEL) staining on fetal nucleated red blood cells (NRBCs) confirmed that 43% to 50% of fetal NRBCs undergo apoptosis [34,35]. Additional studies suggested that fetal cells were being destroyed in the maternal circulation, either by apoptosis [34,36,37], or by another method of programmed cell death [38]. However, subsequent studies have called into question whether fetal hematopoietic cells can account for the entire volume of cffDNA in maternal plasma [39,40].

Physiologic and clinical data suggest that the majority of circulating nucleic acids are derived from the placenta, with some contribution from the fetal hematopoietic system. The discovery of cffDNA in 80% of study subjects by day 28 post-conception implies that the most likely source of cffDNA is the trophoblast, rather than fetal hematopoietic cells, given that the definitive fetoplacental circulation is not established until days 28–30 post-conception [41]. Wataganara et al. provided further evidence for the placental origin of cffDNA when they found that cffDNA could be detected in maternal plasma as long as 11 days after medical abortion, presumably because residual placental tissue continued to contribute to the pool of circulating fetal DNA [23]. Detection of placental DNA in maternal plasma in cases of confined placental mosaicism further supports the placenta as the primary origin of cffDNA in maternal circulation [42,43].

Interestingly, placental volume does not impact cffDNA levels in maternal plasma [44]. Increased concentrations of cffDNA in maternal plasma may reflect placental hypoxia and subsequent increased apoptosis or necrosis of trophoblast [45].

2.3. Cell-free fetal mRNA (cffmRNA)

2.3.1. Characteristics and origin of cffmRNA in maternal plasma

Poon et al. demonstrated that mRNA transcribed from the Y chromosome was detectable in the plasma of women carrying male fetuses [46]. This discovery generated significant excitement because unlike cffDNA in maternal blood, cffmRNA is a gender-independent nucleic acid marker. The detection of cffmRNA in maternal plasma also does not depend on known paternal polymorphisms [47]. Plasma cffmRNA is remarkably stable in peripheral blood [48], perhaps due to its association with placenta-derived microparticles released from cells via a variety of mechanisms, including apoptosis [11]. There is some evidence that these microparticles protect cffDNA and cffmRNA from nuclease degradation [21,22,33,49,50]. When plasma samples from pregnant women were passed through a 0.45 μm filter, there was a significant decrease in human placental lactogen (*hPL*) and beta human chorionic gonadotropin (*ßhCG*) mRNA, as well as mRNA transcripts from the housekeeping gene *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase), providing further support for the hypothesis that cffmRNA in maternal plasma is particle-bound [51].

Through amplification of transcripts from two placental-specific genes, *ßhCG* and *hPL*, the placenta was shown to be the major source of fetal-derived RNA in maternal plasma [51]. The same study found that there was no detectable *hPL* mRNA in umbilical cord plasma samples, suggesting a one-way transfer of *hPL* mRNA from the placenta into the maternal circulation. Maternal plasma is preferable to maternal whole blood for detection of placenta-derived mRNA [52,53]. Placental mRNA transcripts are detectable in maternal plasma by the fourth week of gestation, with a median half-life of 14 min [54]. Like cffDNA, cffmRNA transcripts are rapidly cleared from maternal blood after delivery [48,54]. Fetal hematopoietic cells may also contribute to the pool of cffmRNA [55,56]. A study comparing paired newborn umbilical cord blood samples with antepartum and postpartum maternal whole blood samples suggested that fetal (as opposed to placental) transcripts may also contribute to cffmRNA in maternal circulation [53].

Unlike cffDNA, total cffmRNA levels do not increase throughout gestation. There appears to be an equilibrium of total cffmRNA that does not change over time [47]. Several investigators have demonstrated that individual levels of mRNA transcripts vary depending on the trimester of pregnancy [51,57,58]. Invasive procedures, which are known to transiently increase cffDNA levels, do not affect mRNA levels [59].

2.3.2. Tracking fetal development through gene expression profiling

The systematic identification of placental mRNA markers in maternal plasma through a microarray-based approach was described in 2004 [60], paving the way for possible non-invasive gene expression profiling of the fetus through maternal plasma cffmRNA. Maron et al. performed transcriptional analysis of maternal blood to identify a set of biologically diverse fetal genes [53]. This gene expression microarray analysis compared whole blood mRNA transcripts common to nine term pregnant women and their neonates (via umbilical cord blood), but absent or significantly reduced in postpartum maternal blood. They identified 71 genes in which the mRNA transcript was involved in a developmental process, derived from fetal, placental, or male tissue, associated with a physiological newborn response, or had its expression limited to or highly associated with a fetus or neonate. Twenty-seven developmental genes (neurodevelopmental genes comprised 48% of these), five sensory perception genes (auditory, visual, olfactory), 22 genes involved in fetal physiologic function, and 17 immune defense genes were identified. This transcriptional analysis identified specific gene transcripts that appeared to be associated with a fetus preparing to transition from the in utero environment to extrauterine life.

2.3.3. Non-invasive detection of fetal aneuploidy

Circulating levels of cffmRNA have also been used in the noninvasive diagnosis of trisomy 21. Oudejans et al. were the first to describe a chromosome 21-specific mRNA present in maternal plasma (now called *C21orf105*) [61]. They hypothesized that due to the direct dosage-related difference in expression of chromosome 21-encoded genes, quantification of *C21orf105* in maternal plasma could distinguish between trisomy 21 and euploid pregnancies. However, due to large biological variation between and within individuals, and the low expression profile of *C21orf105*, quantification initially appeared unsuccessful [62–64]. In 2007, a placental-specific mRNA sequence transcribed from a gene located on chromosome 21, *PLAC4*, was identified via a microarray-based approach [65]. Allelic ratios of placental-specific mRNA in maternal plasma were then utilized to detect trisomy 21, with a diagnostic sensitivity of 90% and specificity of 96.5% [65]. The same laboratory later used this placental RNA-SNP allelic ratio method to diagnose trisomy 18 [66], and demonstrated that digital PCR can be used to detect fetal-derived mRNA in maternal plasma. The placental RNA-SNP allelic ratio method may also be theoretically applicable for diagnosis of trisomy 13 [67]. Although gender-independent, the RNA-SNP allelic ratio method is still polymorphism-dependent, which is an important disadvantage. However, to address this limitation, other investigators have described new placental mRNA target polymorphisms that can be analyzed using the RNA-SNP allelic ratio methods [62]. One marker panel has been described that has a combined heterozygosity rate covering up to 95% of the US general population [49].

2.3.4. Detection of complications of pregnancy

CffmRNA may also be useful in diagnosing maternal complications of pregnancy. Ng et al. demonstrated that corticotropin-releasing hormone (*CRH*) mRNA levels were up to 10-fold higher in the plasma of women with preeclampsia compared to normotensive controls [68]. These findings were subsequently confirmed by several groups [69–71]. While levels of *CRH* mRNA increase with increasing gestational age, levels of *CRH* mRNA have not been shown to correlate with severity of preeclampsia [72]. One group found that *CRH* mRNA levels are significantly increased in the plasma of women who subsequently develop preeclampsia, weeks before the onset of clinical symptoms [69].

3. Fetal nucleic acids in amniotic fluid

3.1. Cell-free fetal DNA

3.1.1. Origin

Large amounts of cffDNA were first detected in amniotic fluid in 2001 [7]. A study of 38 amniotic fluid specimens collected for routine indications at 16–20 weeks found that concentrations of cffDNA are 100- to 200-fold greater in amniotic fluid compared to maternal plasma [7]. Zhong et al. utilized real-time PCR amplification to quantify cffDNA in 12 matched maternal plasma and amniotic fluid samples. They found that concentrations of cffDNA were much higher (median 3978 copies/mL) in amniotic fluid compared to maternal plasma (median 96.6 copies/mL) [73].

CffDNA in amniotic fluid originates from a separate pool than cffDNA in maternal plasma and serum. While total cffDNA levels were elevated in the serum of women carrying fetuses affected with Down syndrome, cffDNA in the amniotic fluid of two fetuses with trisomy 21 was not [7]. Makrydimas et al. investigated concentrations of cffDNA in the fetal compared to maternal compartment. They found

that separate physiologic pools of cffDNA contributed to amniotic fluid compared to maternal serum [74]. These investigators examined relative cffDNA concentrations in the amniotic cavity, coelomic cavity, and maternal serum in women undergoing elective first-trimester termination (7–9 weeks' gestation). They showed that cffDNA concentration was highest in the amniotic cavity and lowest in maternal serum. In addition, they argued that the size of cffDNA in maternal circulation (100–300 bp), made it unlikely that this DNA could diffuse through the amniotic membrane into maternal circulation. Lun et al. further developed the concept of separate pools of cffDNA in amniotic fluid and maternal serum through the use of a tissue-specific epigenetic marker associated with the promoter region of the RAS association family 1A (*RASSF1A*) gene [75]. *RASSF1A* is hypermethylated in the placenta but hypomethylated in fetal tissues and in maternal blood cells. By comparing cffDNA in 14 second-trimester amniotic fluid and maternal plasma samples, they found that hypermethylated *RASSF1A* is 30-fold lower in amniotic fluid than in maternal plasma, suggesting that the placenta does not significantly contribute to cffDNA in amniotic fluid.

While the origin of cffDNA in maternal plasma is likely placental, cffDNA in amniotic fluid likely originates directly from the fetus, via shedding through urine, the trachea, fetal blood, or other mechanisms. Thus, cffDNA may provide real-time information about fetal development that cffDNA in maternal plasma cannot.

Recent improvements in cffDNA extraction methods improved the yield of cffDNA from amniotic fluid supernatant [76,77]. Gestational age, storage time, and fetal karyotype all influence cffDNA fragmentation patterns [78,79]. Notably, the quantity and integrity of cffDNA is significantly lower in frozen than in fresh samples, and in aneuploid than euploid samples, after adjusting for gestational age. No linear relationship was observed between time in frozen storage and concentrations of cffDNA in amniotic fluid [79]. Winter et al. found that high-mobility group protein HMGA2, which is primarily expressed by embryonic and fetal cells, is bound to cffDNA in amniotic fluid. This finding has significance for improving the yield of cffDNA from supernatant via immunoprecipitation techniques [80].

3.2. Cell-free fetal mRNA

3.2.1. Characteristics

Successful isolation of cffmRNA from amniotic fluid has resulted in discovery of new information about real-time fetal development and physiology [8,81]. Larrabee et al. demonstrated, through filtration of centrifuged and uncentrifuged amniotic fluid from a patient with polyhydramnios, that cffmRNA in amniotic fluid is particle-bound, similar to cffmRNA in maternal plasma [82]. It is currently unknown whether cffmRNA in amniotic fluid plays a functional role, or whether it is most useful as a reflection of functional development of fetal tissues in direct contact with amniotic fluid. Just as epigenetic studies of cffDNA in amniotic fluid demonstrate very little contribution from the placenta [75], the limited detection of placental genes in gene expression microarrays suggests that the placenta does not contribute significantly to cffmRNA in amniotic fluid.

3.2.2. Gene expression analysis

Global gene expression analysis of cffmRNA in amniotic fluid was first reported by Larrabee et al. in 2005 [8]. Amniotic fluid was obtained from women between 20 and 32 weeks of gestation undergoing therapeutic amnioreduction for polyhydramnios due to twin-to-twin transfusion syndrome or hydrops fetalis. Four cases were compared to a pooled second-trimester control sample. The mRNA was then analyzed using Affymetrix U133A arrays. Statistically higher expression of *aquaporin-1*, a gene involved in water transport, was observed in fetuses with TTTS, suggesting that this gene may contribute to the amniotic fluid imbalances noted in TTTS. The same study also examined gene expression differences by gestational age, finding that more surfactant genes were expressed at later gestational ages, consistent with known patterns of fetal lung maturation. More salivary and tracheobronchial gene transcripts were found at later gestational ages, as well as fewer keratin gene transcripts, reflecting epithelial maturation.

Hui et al. sought to determine which mRNA transcripts are ubiquitously present in the amniotic fluid supernatant of euploid fetuses in the second trimester, in order to develop a normal second trimester amniotic fluid core transcriptome [83]. Ingenuity® Pathways Analysis was utilized to infer the major physiologic functions and pathways critical to mid-trimester human fetal development. Fetal organ specificity was examined using the GNF Gene Expression Atlas as well as manual literature searching. This investigation utilized gene expression data from 12 euploid second-trimester amniotic fluid samples. Four hundred seventy well-annotated genes were present in 12/12 samples. Twenty-three highly organ-specific transcripts were identified; one-third of these were expressed by the fetal brain. Putative organ sources of cffmRNA included the brain, spinal cord, lung, pancreas, liver, tongue, blood, heart, and kidney. These findings imply that a fetal gene expression panel to evaluate normal organ system function is feasible.

A recent proof-of-concept study sought to assess fetal functional gene expression in amniotic fluid supernatant obtained from 19 euploid fetuses in the second trimester, utilizing standardized NanoArray PCR (SNAP) [84]. SNAP uses internal standard (IS) sequences to measure relative abundance of a gene transcript. Statistically significant differences in gene expression as a function of advancing gestational age and fetal gender were noted, suggesting that SNAP and other transcriptomic analyses hold great potential for understanding fetal development in real time.

3.2.3. Differential gene expression in aneuploid fetuses

In 2009, a functional genomic approach was utilized to study the development of fetuses with Down syndrome [81]. This study compared gene expression in fetuses with trisomy 21 to those with normal chromosomes at matched gestational ages. Microarray analysis identified 414 probe sets, corresponding to 311 annotated genes differentially expressed in trisomy 21, only five of which were physically located on chromosome 21. Gene set enrichment analysis identified a single chromosomal band (21q22) containing genes that were up-regulated as a group. The differentially-regulated genes were analyzed using DAVID (Database for Annotation, Visualization, and Integrated Discovery). This functional analysis revealed that oxidative stress, ion transport, and immune and stress response were the processes that were significantly disrupted in fetuses with Down syndrome.

A subsequent study compared cffmRNA from the second-trimester amniotic fluid supernatant of five fetuses with trisomy 18 to six gestational-age-matched euploid controls [12]. Hybridization of mRNA to Affymetrix U133 Plus 2.0 assays revealed 352 probe sets, corresponding to 251 annotated genes, that were differentially expressed between trisomy 18 and euploid samples. Only seven of these differentially expressed genes were actually located on chromosome 18. One of the seven genes located on chromosome 18 was *ROCK1*, a gene involved in valvuloseptal and endocardial cushion formation. *ROCK1* was also found to be overexpressed in trisomy 21 [81]. Functional analyses revealed that ion transport, cell-mediated immunity, DNA repair, G-protein mediated signaling, kinase function, and glycosylation were all disrupted in trisomy 18. Significant down-regulation of genes involved in adrenal development was noted, as was up-regulation of genes associated with cardiovascular development, endocrine function, lipid metabolism, and molecular transport.

4. Cell-free fetal nucleic acids in other maternal body fluids

Cff nucleic acids have been identified in other maternal biofluids, such as urine, CSF, and peritoneal fluid. Through isolation of male-

specific DNA sequences in the urine of women pregnant with male fetuses, Botezatu et al. found that the human glomerular barrier is permeable to DNA molecules of sufficient size to be analyzed by PCR [3]. Shortly thereafter, Al-Yatama et al. demonstrated that nested PCR amplification studies could be used to detect Y-chromosome-specific fetal DNA in urine, although the sensitivity and specificity of this technique were better for maternal plasma than for urine [4]. Investigation of cffDNA fragmentation patterns in maternal plasma and urine revealed that fetal DNA fragments in maternal urine are smaller than those in maternal plasma, providing a potential explanation for the lower sensitivity and specificity of detection of cffDNA in maternal urine [31]. Cell-free fetal DNA has also been detected in maternal CSF and peritoneal fluid. Analysis of CSF from 39 women undergoing spinal anesthesia in the third trimester found that *DYS1* gene sequences were detectable in four of 26 samples from women carrying at least one male fetus and 0 of 13 samples from women carrying only a female fetus [40]. Cioni et al. reported the detection of Y-chromosome specific sequences in the peritoneal fluid of a primigravida undergoing surgical intervention for ovarian torsion at 12 weeks' gestational age; the patient went on to deliver a male fetus [6]. Makrydimas et al. also identified cffDNA in peritoneal fluid, reporting a decrease in concentration of cffDNA from amniotic cavity, to maternal peritoneal cavity, to maternal serum [74].

5. Clinical applications

5.1. CffDNA in maternal serum and plasma

5.1.1. Fetal sex

First trimester fetal sex determination is important for prenatal diagnosis of congenital adrenal hyperplasia (CAH) and for X-linked disorders. Noninvasive early fetal sex determination would limit unnecessary maternal dexamethasone therapy to reduce masculinization of a female fetus in cases of CAH, and reduce the risk of miscarriage associated with invasive prenatal diagnosis for X-linked disorders [85,86]. Amplification of *SRY* permits accurate identification of fetal gender, although accuracy increases with increasing gestational age. Individual studies report 80% accuracy of gender identification by seven weeks and as high as 100% accuracy by nine weeks of gestation [86–88]. A study utilizing the deleted in azoospermia (*DAZ*) region of the long arm of the Y chromosome demonstrated detection of fetal gender by the fifth week of gestation, with 100% accuracy by eight weeks [89]. A recent meta-analysis of 57 selected studies representing 3524 male-bearing and 3017 female-bearing pregnancies demonstrated that Y-chromosome-specific sequences were detected in maternal blood with 95.4% sensitivity and 98.6% specificity overall, with significant interstudy heterogeneity. Real-time quantitative PCR outperformed conventional PCR, and testing after 20 weeks (sensitivity, 99.0%; specificity, 99.6%) outperformed testing prior to 7 weeks (sensitivity, 74.5%; specificity, 99.1%). Sensitivity and specificity of testing increased steadily from 7 to 20 weeks. Tests using urine and tests performed before 7 weeks' gestation were unreliable [90]. Direct-to-consumer genetic tests in which dried maternal blood spots are used to detect fetal Y-specific (*DYS1*) sequences [91] are available to the public in pharmacies and via the internet without regulation. The accuracy of these tests is unknown. The ready availability of fetal gender tests has raised important ethical considerations [92].

5.1.2. Noninvasive fetal RhD genotyping

Lo et al. were the first to describe noninvasive fetal RhD genotyping using maternal blood. This study demonstrated more reliable diagnosis of fetal RhD genotype in the second and third trimesters; the first trimester demonstrated only a 78% concordance rate [93]. Improved real-time PCR (RT-PCR) techniques subsequently increased the sensitivity of the RhD assay in the first trimester to as high as 100% [94]. Cardo et al. recently utilized quantitative PCR techniques to diagnose fetal RhD genotype from first trimester maternal plasma samples, reporting a sensitivity of 100% and specificity of 93%, with overall diagnostic accuracy of 97% [95]. False positive cases are most often due to RhD negative black Africans who actually have an RhD pseudosequence that is intact but nonfunctional, called RhD ψ, or a hybrid RHD-CE-D^s gene. RT-PCR amplification with specially-designed sequence-specific primer pairs has subsequently been utilized to distinguish RhD from RhD ψ, with reported 99.6–100% accuracy [96,97]. RhD genotyping has been increasingly incorporated into routine prenatal care in France, Denmark, the Netherlands, and the United Kingdom, and many believe that this application is now appropriate for universal clinical application [98].

5.1.3. Single gene disorders

In addition to RhD genotyping, cffDNA has also been utilized in the prenatal detection of multiple other monogenic disorders. While the accuracy of *RHD* DNA detection from maternal plasma approaches nearly 100%, the noninvasive diagnosis of single gene disorders characterized by more subtle genetic differences between the maternal and fetal DNA sequences has posed more of a challenge. A mutation-specific RT-PCR assay was successfully used to detect the most common Southeast Asian β-thalassemia mutation in 2002 [99]. However, over 200 β-thalassemia mutations have been described, many of which are point mutations [100]. The detection of single-base differences in fetal DNA with RT-PCR has proven difficult, due to the lack of absolute specificity of allele-specific primers [101] and the low concentration of fetal DNA in maternal plasma. A matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry (MS) method and a single allele base extension reaction (SABER) MS method have been successfully utilized to detect paternally-inherited β-thalassemia point mutations in maternal plasma [102]. Li et al. described the use of a peptide nucleic acid clamp specific for the maternal allele to suppress unwanted PCR amplification of this allele, thereby increasing the accuracy of detection of a paternally-inherited β-thalassemia point mutation [103].

Both digital PCR and gel electrophoresis for size fragmentation of maternal versus fetal cfDNA have been applied in the NIPD of single gene disorders. Lun et al. have described the use of a digital PCR-based relative mutation dosage approach, coupled with a digital nucleic acid size selection strategy, to enrich fetal DNA for the diagnosis of β-thalassemia and hemoglobin E mutations [104]. Tsui et al. later applied the same digital PCR-based relative mutation dosage approach to the prenatal diagnosis of an X-linked disorder, hemophilia [105]. These investigators were able to correctly classify fetal hemophilia mutations in 12 maternal plasma samples obtained from 7 hemophilia carriers pregnant with male fetuses. The discovery that cffDNA fragments are significantly smaller than maternally-derived DNA fragments prompted the utilization of gel electrophoresis to isolate these shorter DNA fragments [103,106,107]. This approach has been successfully employed to diagnose single gene disorders characterized by point mutations, such as achondroplasia [108,109] and β-thalassemia [103,110,111].

Recent investigations of the use of PCR for NIPD of alpha-thalassemia, specifically Hemoglobin Bart's, demonstrated the need for ongoing research to improve the sensitivity and precision of these techniques. One investigation failed to reliably diagnose Hemoglobin Bart's using real-time quantitative PCR [112]. Another investigation utilized qualitative fluorescence PCR to exclude Hemoglobin Bart's in 10/30 pregnancies, via detection of nondeleted paternally inherited fetal alleles [113].

The successful diagnosis of Huntington disease via cffDNA in maternal plasma has been described only in case reports [63,114,115]. It has been found to be more accurate when the fragment of the *IT15* gene containing the CAG repeat is targeted. The indirect analysis, utilizing a polymorphic microsatellite located in exon 1 of the

IT15 gene, was not able to accurately diagnose fetuses [115]. Currently, the ability to diagnose Huntington disease via maternal blood is limited to those pregnancies in which the father of the fetus is affected. The use of allele-specific PCR to diagnose fetal conditions in the setting of known paternally-inherited mutations has also been described for cystic fibrosis [101,116] and myotonic dystrophy [117].

5.1.4. Non-invasive diagnosis of fetal aneuploidy

The detection of fetal aneuploidy has posed a significant technical challenge compared to the previously described applications because there is no unique fetal mutation. Prior investigations demonstrated elevated cffDNA in maternal plasma and serum in cases of fetal trisomies 21 [32,118,119] and 13, but not in cases of trisomy 18 [120]. Two studies provided conflicting results, finding no association between elevated cffDNA levels in maternal plasma/serum and aneuploidy [118,121]. The promise of an association between elevated cffDNA and aneuploidy led to the investigation of second-trimester cffDNA levels as an additional serum screening marker. Incorporating cffDNA measurements increased the detection rate for Down syndrome from 81 to 86% when added to the quadruple screen panel [122].

The use of epigenetic markers is another proposed strategy for prenatal diagnosis. Epigenetic modifications are alterations in phenotype or gene expression without changes in the actual DNA sequence, such as DNA methylation [123]. The discovery of differentially methylated regions (DMRs) between fetal DNA and maternal tissues provided the basis for an epigenetic approach to prenatal diagnosis [124]. This approach has the advantage of allowing for the detection of both maternally and paternally inherited fetal alleles [124]. The epigenetic–genetic chromosome dosage approach utilizing the putative promoter of the holocarboxylase synthetase (*HLCS*) gene on chromosome 21 was demonstrated to correctly diagnose trisomy 21 in both male and female fetuses [125]. *Maspin*, the first universal fetal DNA marker in maternal plasma, is a placental epigenetic marker that is methylated in maternal leukocytes and hypomethylated in the placenta [126]. The discovery that *maspin*, also known as the *SERPINB5* gene (located on chromosome 18) had different methylation patterns in the placenta compared to maternal blood cells led to the development of a strategy utilizing epigenetic allelic ratios to diagnose trisomy 18 in the fetus [66,126]. This discovery prompted the search for fetal-specific epigenetic markers on chromosome 21 [127–129]. Of 114 studied genomic regions on chromosome 21, 22 DMRs have been identified [128]. A recent investigation by Papageorgiou et al. utilized methylated DNA immunoprecipitation methodology (MeDiP) and real-time quantitative PCR to achieve noninvasive prenatal detection of trisomy 21 in 14 cases [130]. The investigators tested methylation ratios for fetal-specific DMRs present in maternal peripheral blood and were also able to correctly diagnose 26 euploid controls. The recently developed MeDiP technique [131,132] overcomes two of the major limitations of prior techniques employed for epigenetic analysis. There is no need to use DMRs containing a restriction site, [127,130], nor to use the methylation-sensitive enzyme sodium bisulfate, which leads to DNA degradation [133].

The limitations of RT-PCR in detecting aneuploidy have also been addressed by digital PCR and microfluidics [134]. Digital PCR does not depend on allelic distribution or gender and is able to detect fetal DNA signals even in the presence of contaminating maternal DNA or mosaicism. Through the utilization of microfluidic valves, one sample can be divided into hundreds to thousands of individual reactions, each containing a single template copy. The number of individual positive PCR reactions is counted and a quantitative assessment of the presence of the target region is made [134,135]. The thousands of individual reactions facilitate recognition of very small changes in the amount of DNA, thus permitting the detection of the 1.5-fold difference in input DNA concentration occurring in trisomy 21 against a background of abundant maternal DNA transcripts in maternal plasma [134,136].

Fan and Quake utilized a microfluidic chip to examine human genomic DNA from a trisomy 21 cell line compared to DNA from a normal cell line [137]. They compared the dosage of an amyloid gene sequence on chromosome 21 to that of the *GAPDH* locus on chromosome 12 and demonstrated that digital PCR could reliably discriminate between normal and aneuploid samples, even if aneuploid material comprised only 10% of the total material examined. This demonstrated that digital PCR was more precise and sensitive than RT-PCR or fluorescent QF-PCR (later confirmed by Lun et al. [138]), and suggested that digital PCR could be suitable for detection of fetal aneuploidy via cffDNA in maternal blood. The same investigators later utilized microfluidic digital PCR to accurately identify all cases of fetal trisomy in 24 amniocentesis and 16 chorionic villus samples [139].

Lo et al. also utilized digital PCR to diagnose aneuploidy via cffDNA and cffmRNA [136]. They described two digital PCR strategies for NIPD of fetal aneuploidy. The first was based on the *PLAC4* mRNA SNP approach described in Section 2.3.3, and utilized maternal plasma of women carrying fetuses affected with trisomy 21. The second strategy used a digital relative chromosome dosage approach to evaluate the dosage of a locus on chromosome 21 compared to a locus on chromosome 1; this method was able to detect fetal aneuploidy in a mixture of euploid and aneuploid DNA with as low as 25% trisomic DNA.

As digital PCR was being developed, significant changes were occurring in DNA sequencing technology. Massively parallel shotgun sequencing (MPSS) of cffDNA in maternal plasma, also known as next-generation sequencing, was first described in 2008 by two independent reports published two months apart [19,140]. Since those initial reports, MPSS of cffDNA in maternal plasma has been used to diagnose trisomies 21, 18 and 13 with high sensitivity and specificity in five independent large clinical trials [141–145].

Chiu et al. were the first to attempt to validate the clinical efficacy and feasibility of MPSS of cffDNA in maternal plasma for diagnosis of trisomy 21 [145]. These investigators utilized two-plex sequencing to detect trisomy 21 in 314 plasma samples from high-risk pregnant women, reporting 100% sensitivity and 97.9% specificity. An eight-plex sequencing protocol was tested on 753 maternal plasma samples, with a 79.1% detection rate of trisomy 21, and 98.9% specificity. The authors concluded that the use of MPSS to rule out fetal trisomy 21 would allow pregnant women to avoid 98% of invasive diagnostic procedures. Ehrich et al. used MPSS for noninvasive detection of trisomy 21 in 449 maternal plasma samples, reporting 100% sensitivity and 99.7% specificity [141]. Sehnert et al. sought to detect not only fetal trisomy 21 but also trisomy 18, utilizing MPSS of cffDNA from 119 maternal plasma samples [142]. These investigators reported 100% correct classification of trisomy 21 (13 of 13) and trisomy 18 (eight of eight). Chen et al. also utilized MPSS of cffDNA from 392 maternal plasma samples to evaluate for trisomies 13 and 18 [143]. Using a non-repeat-masked reference human genome and a bioinformatics approach to correct GC content bias, they correctly identified 25/25 fetuses with trisomy 13 (98.9% specificity) and 34/37 fetuses with trisomy 18 (98% specificity). Although the aforementioned results were promising, these studies had relatively small sample sizes, DNA sequencing performed at non-CLIA-certified laboratories, and long turnaround time that did not simulate clinical practice. Palomaki et al. undertook a clinical validation study to overcome the shortcomings of previous studies [144]. These investigators evaluated the ability of next-generation sequencing to identify trisomy 21 in 212 affected pregnancies and 1484 matched euploid controls, reporting a 98.6% detection rate with a 0.2% false-positive rate. The same investigators later evaluated the accuracy of MPSS for detection of trisomies 13 and 18, reporting a detection rate of 100% for trisomy

18 and 91.7% for trisomy 13 [146]. False positive rates were 0.28% and 0.97%, respectively.

It remains unclear whether MPSS of cffDNA in maternal plasma can truly be used to diagnose aneuploidy, or whether it should be used to triage pregnancies at high risk for trisomy 21 for subsequent invasive diagnostic procedures [145]. The International Society of Prenatal Diagnosis (ISPD) released a position statement on aneuploidy screening in 2011, stating that "non-invasive approaches to screening and diagnosis through the analyses of fetal cells or nucleic acids in maternal circulation…have not yet been validated in clinical trials and are therefore not ready for clinical use [147]." In a subsequent response to Palomaki et al.'s 2011 report, the ISPD released a rapid response position statement stating that massively parallel sequencing of cffDNA in maternal plasma to detect trisomy 21 "constitutes an advanced screening test" and that "confirmation of MPS positive results through invasive testing would still be required [148]." Table 1 depicts the availability of noninvasive prenatal diagnostic tests using cell-free fetal nucleic acids in the United States, Europe, and China.

5.1.5. Detection of complications of pregnancy

Given that the placenta is the primary tissue source of circulating fetal DNA, it is intuitive that changes in cffDNA levels are observed in abnormal placentation. Elevations in cffDNA have been demonstrated in cases of placenta previa, accreta, and increta [149,150]. Preeclampsia, another disease associated with abnormal trophoblast invasion, is also associated with elevated levels of cffDNA in maternal plasma. Significant increases in cffDNA in maternal plasma have been noted in preeclamptic women compared to normotensive pregnant controls [151–153]. This increase may precede the development of clinical symptoms [154,155]. An initial elevation of cffDNA in the plasma of women who will go on to develop preeclampsia occurs at 17–28 weeks, and a second elevation is noted approximately 3 weeks prior to the onset of clinical symptoms [156]. Maternal DNA levels are also elevated in women with preeclampsia [157,158]. The concentration of maternal and fetal DNA in maternal plasma correlates positively with disease severity; women with hemolysis, elevated liver enzymes, and low platelets (HELLP) have higher levels of cffDNA than preeclamptic women without HELLP. Both have higher levels than normotensive gestational age-matched controls [158].

The data are conflicting on levels of cffDNA in maternal circulation in cases of fetal intrauterine growth restriction (IUGR). In one study, abnormal uterine artery Doppler studies and elevated cffDNA levels in maternal plasma were associated with the development of IUGR [159]. However, another study comparing plasma of women with preeclampsia, to that of women with fetuses with IUGR, to that of gestational age-matched controls, found no increase in cffDNA in the plasma of pregnant women with fetal IUGR [160]. Elevations in cffDNA have also been noted in hyperemesis gravidarum [161], polyhydramnios [162], and preterm delivery [163], although a recent study found that cffDNA levels in maternal plasma did not predict preterm delivery in women with a short cervix at 22–24 weeks [164].

5.2. CffDNA in amniotic fluid

Two studies have investigated utilizing cffDNA in amniotic fluid to produce a rapid "molecular karyotype." Larrabee et al. were the first group to utilize fetal DNA from amniotic fluid supernatant for prenatal molecular diagnosis [9]. Using cffDNA from 28 amniotic fluid supernatant samples for comparative genomic hybridization (CGH) microarray analysis, they were able to successfully identify fetal gender, as well as whole-chromosome gains and losses on chromosomes X and 21. Miura et al. used a targeted microarray-based CGH panel spotted with bacterial artificial chromosome (BAC) clones of chromosomes 13, 18, 21, X and Y to perform successful molecular karyotyping for 12 of 13 fetuses within 5 days [10]. One false-negative result was obtained for a fetus with a balanced translocation. After Lapaire et al. described technical advances to increase the yield and quality of cffDNA extracted from amniotic fluid [76], they utilized array CGH to correctly detect whole chromosome aneuploidy in eight of nine cases, only missing one case of triploidy [165]. The advantage of using cffDNA in amniotic fluid compared to DNA from cultured cells is its turn around time. This technique has already transitioned to clinical care.

6. Future directions

As a result of advanced parental ages, obesity, and other environmental exposures, infertility and the utilization of assisted reproductive technologies are becoming increasingly common. NIPD of fetal aneuploidy is a rapidly evolving area. As of late 2011, MPSS of cffDNA in maternal plasma is being offered on a commercial basis in China and the United States. Its clinical role in prenatal screening or diagnosis remains to be determined.

The cost and complexity of MPSS may limit its broad implementation for NIPD of fetal aneuploidy. One disadvantage of MPSS is that it generates large amounts of unutilized sequencing data. Recent investigations have tried to address this shortcoming, via selective sequencing of cffDNA in maternal plasma. Lo et al. performed a proof-of-concept study utilizing paired-end MPSS to selectively sequence SNPs identified from information about the paternal genotype and maternal haplotype, in order to construct a genome-wide genetic map of the fetus [166]. Their findings suggest that MPSS may be used to scan the fetal genome for known paternal and maternal polymorphisms, enabling targeted and efficient NIPD. Sparks et al. utilized a novel, highly multiplexed assay called digital analysis of selected

Table 1
Global availability of noninvasive prenatal diagnostic tests using cell-free fetal nucleic acids.

Test	Status of NIPD[a] as of early 2012		
	U.S.	Europe	China
Fetal RhD genotyping (PCR)[b]	Available/CLIA[c] Academic and commercial	Available/academic	Not applicable due to low incidence of RhD negative women
Fetal sex determination (PCR)	Available/DTC[d] Not available/CLIA	Available/academic	Available/commercial
Fetal aneuploidy (MPSS)[e]	Available/CLIA Commercial	Available/academic and commercial	Available/academic and commercial
Array CGH[f] on amniotic fluid	Available/CLIA/academic and commercial	Available/academic and commercial	Available/academic and commercial

[a] Noninvasive prenatal diagnosis.
[b] Polymerase chain reaction.
[c] Clinical Laboratory Improvement Amendments.
[d] Direct to Consumer.
[e] Massively parallel shotgun sequencing.
[f] Comparative genomic hybridization.

regions (DANSR) to selectively sequence specific fragments of cffDNA for evaluation of fetal trisomy [167]. Using DANSR assays for loci on chromosomes 21 and 18, these investigators were able to correctly classify all cases of T21 (39) and T18 (7) in the 298 pregnant women studied. Selective sequencing of cffDNA in maternal plasma takes advantage of the sensitivity of next-generation sequencing, while improving its efficiency and presumably lowering its cost.

While the diagnosis of aneuploidy via next-generation sequencing has been a recent focus, advances in molecular techniques will likely allow for greater precision and utilization of NIPD for single-gene disorders. Another future direction may be the noninvasive prenatal identification of fetal polymorphisms known to be associated with complications of pregnancy such as preeclampsia and gestational diabetes. Two different single nucleotide polymorphisms (SNPs) in the fetal endoplasmic reticulum aminopeptidase 2 (*ERAP2*) gene have been reported to be associated with increased risk for preeclampsia in an African-American population, a New Zealand/Australian population, and a Norwegian population [168]. Paternally-inherited fetal polymorphisms in the insulin-like growth factor-2 gene (*IGF2*) were found to be associated with increased maternal glucose concentrations in pregnancy [169]. To date, however, such polymorphisms have primarily been identified in neonatal blood rather than via cffDNA in maternal blood. As more fetal polymorphisms associated with maternal complications of pregnancy are discovered, identification of these polymorphisms via cffDNA in maternal plasma may be utilized to help predict such complications.

Recent work on the fetal transcriptome presents an exciting opportunity to do more than diagnose an abnormality; this represents the first opportunity to track fetal development in real-time in both normal and pathologic states [83]. The unexpected discovery of fetal brain transcripts in amniotic fluid may facilitate new ways of monitoring fetal nervous system development. Comparisons of the normal second and third trimester amniotic fluid transcriptome to the fetal mRNA transcriptome present in disease states (both maternal and fetal) ultimately may allow for the practice of personalized fetal genomic medicine.

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